

~~ALLERGENIC PROTEIN AND PEPTIDES FROM~~
~~HOUSE DUST MITE AND USES THEREFOR~~
A HOUSE DUST MITE ALLERGEN, DER F V.II, AND
USES THEREOF

5 Background of Invention

Approximately 10% of the population become hypersensitized (allergic) upon exposure to antigens from a variety of environmental sources. Those antigens that induce immediate and/or delayed types of hypersensitivity are known as allergens (King, T.P., (1976) Adv. Immunol., 23:77-105). These include products of grasses, trees, weeds, animal dander, insects, food, drugs, and chemicals. Genetic predisposition of an individual is believed to play a role in the development of immediate allergic responses (Young, R. P. et al., (1990) Clin. Sci., 79:19) such as atopy and anaphylaxis whose symptoms include hay fever, asthma, and hives.

The antibodies involved in atopic allergy belong primarily to the IgE class of immunoglobins. IgE binds to basophils, mast cells and dendritic cells via a specific, high-affinity receptor FcεRI (Kinet, J.P., (1990) Curr. Opin. Immunol., 2:499-505). Upon combination of an allergen acting as a ligand with its cognate receptor IgE, FcεRI bound to the IgE may be cross-linked on the cell surface, resulting in physiological manifestations of the IgE - allergen interaction. These physiological effects include the release of, among other substances, histamine, serotonin, heparin, chemotactic factor(s) for eosinophilic leukocytes and/or leukotrienes C4, D4, and E4, which cause prolonged constriction of bronchial smooth muscle cells (Hood, L.E. et al., Immunology (2nd ed.), The Benjamin/Cumming Publishing Co., Inc. (1984)). Hence, the ultimate consequence of the interaction of an allergen with IgE is allergic symptoms triggered by the release of the aforementioned mediators. Such symptoms may be systemic or local in nature, depending on the route of entry of the antigen and the pattern of deposition of IgE on mast cells or basophils. Local manifestations generally occur on epithelial surfaces at the site of entry of the allergen. Systemic effects can induce anaphylaxis (anaphylactic shock) which results from IgE-basophil response to circulating (intravascular) antigen.

Studies with purified allergens have shown that about 80% of patients allergic to the mite *Dermatophagoides pteronyssinus* produce IgE reactive to Der p I and Der p II (Chapman M.D. et al., J. Immunol. (1980) 125:587-92; Lind P., J. Allergy Clin. Immunol. (1985) 76:753-61; Van der Zee J.S. et al., J. Allergy Clin. Immunol. (1988) 81:884-95). For about half of the patients, these specificities constitute 50% of the IgE antimite antibody. The allergen Der p III, recently identified as trypsin, (Stewart G.A. et al., Immunology (1992) 75:29-35) reacts with a similar or higher frequency (Stewart G.A. et al., supra; Ford S.A. et al., Clin. Exp. Allergy (1989) 20:27-31). However, in the only quantitative study performed to date, the investigators reported the level of IgE binding to be considerably less than Der p I. Electrophoretic techniques (Ford S.A. et al., supra; Bengtsson A. et al., Int. Arch. Allergy

Appl. Immunol. (1986) 80:383-90; Lind P. et al., Scand. J. Immunol. (1983) 17:263-73; Tovey E.R. et al., J. Allergy Clin. Immunol. (1987) 79:93-102) have shown that most sera recognize other allergens. For example, in the study of Ford et al. (supra) Western blotting showed 8 sera reacting with 1-2 bands, 6 with 3-6 and 3 with a greater number including one with at least 13. In another study, Baldo et al. (Adv. Bioscience (1989) 4:13-31) report the finding of components at Mr 30, 26, 25K reacting with 50% of sera. To determine the importance of particular specificities in the allergic reactions, purified allergens would be required for quantitative IgE binding tests and to examine the frequency and lymphokine profile for T cell reactivity.

Treatment of patients with sensitivity to house dust mites by administration of increasing doses of house dust extracts has the drawbacks of potential anaphylaxis during treatment and the possible necessity of continuing therapy over a period of several years to build up sufficient tolerance that results in significant diminution of clinical symptoms. A therapeutic composition and method of therapy which avoids these problems would be beneficial.

Summary of the Invention

This invention provides isolated nucleic acids encoding peptides having at least one biological activity of Der p VII or Der f VII, protein allergens of the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. Preferred nucleic acids are cDNAs having a nucleotide sequence shown in Figure 3A and 3B (SEQ ID NO: 1) (Der p VII) and Figure 6A and 6B (SEQ ID NO: 6) (Der f VII). The invention also pertains to peptides encoded by all or a portion of such cDNAs (SEQ ID NO: 1 and SEQ ID NO: 6) and having at least one biological activity of Der p VII or Der f VII. Also contemplated are isolated nucleic acids which hybridize under high stringency conditions (e.g., equivalent to 20-27°C below T_m and 1M NaCl) to a nucleic acid having a nucleotide sequence shown in Figure 3A and 3B (SEQ ID NO: 1) or Figure 6A and 6B (SEQ ID NO: 6) or which encodes a peptide comprising all or a portion of an amino acid sequence of Figure 3A and 3B (SEQ ID NO: 2)(Der p VII) or Figure 6A and 6B (SEQ ID NO: 7)(Der f VII). Nucleic acids which encode peptides having an activity of Der p VII or Der f VII and having at least 50% homology with a sequence shown in Figure 3A and 3B (SEQ ID NO: 2)(Der p VII) or Figure 6A and 6B (SEQ ID NO: 7)(Der f VII) are also featured. Peptides having a Der p VII or Der f VII activity produced by recombinant expression of a nucleic acid of the invention, and peptides having a Der p VII or Der f VII activity prepared by chemical synthesis are also featured by this invention. Preferred peptides have the ability to induce a T cell response, which may include T cell stimulation (measured by,

for example, T cell proliferation or cytokine secretion) or T cell nonresponsiveness (i.e., contact with the peptide or a complex of the peptide with an MHC molecule of an antigen presenting cell induces the T cell to become unresponsive to stimulatory signals or incapable of proliferation). Other preferred peptides, either apart from or in addition to the ability to induce a T cell response, have the ability to bind the dust mite specific IgE of dust mite-allergic subjects. Such peptides are useful in diagnosing sensitivity to dust mite in a subject. Still other peptides, either apart from or in addition to the ability to induce a T cell response, have a significantly reduced ability to bind dust mite-allergic IgE. Such peptides are particularly useful as therapeutic agents.

Other preferred peptides comprise an amino acid sequence shown in Figure 3A and 3B (SEQ ID NO: 2) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 7) (Der f VII). In one embodiment, peptides having a Der p VII or Der f VII activity and comprising a portion of the amino acid sequence of Figure 3A and 3B (SEQ ID NO: 2) or Figure 6A and 6B (SEQ ID NO: 7) are featured. Such peptides are at least about 8-30 amino acids in length, preferably about 10-20 amino acids in length, and most preferably about 10-16 amino acids in length.

Another aspect of the invention features antibodies specifically reactive with a peptide having a Der p VII or Der f VII activity. A peptide having an activity of Der p VII or Der f VII can be used in compositions suitable for pharmaceutical administration.

Such compositions can be used in a manner similar to dust mite extracts to treat or prevent allergic reactions to a dust mite allergen in a subject. Nucleic acids of the invention and peptides having an activity of Der p VII or Der f VII can also be used for diagnosing sensitivity in a subject to a dust mite allergen.

Brief Description of the Drawings

Fig. 1 shows the binding frequency of IgE from allergic sera with λ gt11-HD6 plaques.

Fig. 2 shows the reactivity of IgE and rabbit anti-house dust mite antibody to purified glutathione-S-transferase fusion product of the HD6 insert cloned into pGEX-1.

Fig. 3A and Fig. 3B is the nucleotide sequence and deduced amino acid sequence of Der p VII clone HD6.

Fig. 4 shows extracts of house dust mites electrophoresed on a 8-18% SDS-PAGE, electroblotted onto nitrocellulose and reacted with pooled allergic serum absorbed with lysates from *E. coli* containing a pGEX-1 vector control (*lane 1*) or pGEX-1 HD6 (*lane 2*).

Fig. 5 shows the reactivity of affinity purified anti-HD6 antibodies to *D. pteronyssinus* extracts. Rabbit antibodies were affinity purified on nitrocellulose and used to probe a Western blot of mite extracts, electrophoresed on 8-18% SDS-PAGE and developed with ¹²⁵I-protein A.

Fig. 6A and Fig. 6B is the nucleotide sequence and deduced amino acid sequence of Der f VII.

Fig. 7A, 7B, 7C, 7D, and 7E is a comparison of the nucleotide sequence and deduced amino acid sequence of Der f VII and Der p VII. Dots indicate a consensus in nucleotide sequence between Der f VII and Der p VII. Nucleotide bases which differ between Der f VII and Der p VII are indicated, along with any corresponding amino acid differences.

Detailed Description of the Invention

This invention pertains to isolated nucleic acids encoding peptides having at least one biological activity of Der p VII or Der f VII, allergens of the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively. Preferably, the nucleic acid is a cDNA comprising a nucleotide sequence shown in Figure 3A and 3BA and 3B (SEQ ID NO: 1) (Der p VII) or Figure 6A and 6BA and 6B (SEQ ID NO:6) (Der f VII).

The cDNA shown in Figure 3A and 3BA and 3B (SEQ ID NO:1) encodes a Der p VII peptide which includes a 17 amino acid leader sequence encoded by base 68 through base 118. This leader sequence is not found in the mature Der p VII protein, which is encoded by bases 119 through 715. The deduced amino acid sequence of Der p VII based on this cDNA is also shown in Figure 3A and 3B (SEQ ID NO: 2). The cDNA encodes a 198 residue mature peptide having a predicted molecular weight of 22,177 Da, no cysteines and a single potential N-linked glycosylation site. A host cell transfected with an expression vector containing a nucleotide sequence encoding Der p VII was deposited under the Budapest Treaty with the American Type Culture Collection on July 6, 1993 and assigned accession number 69.348.

The cDNA shown in Figure 6A and 6B (SEQ ID NO: 6) encodes a Der f VII peptide. Der f VII peptide is encoded by bases 43 through 681 of this cDNA sequence. The deduced amino acid sequence of Der f VII based on this cDNA is shown in Figure 6A and 6B (SEQ ID NO: 7). Similar to Der p VII, this Der f VII peptide may contain a leader sequence not found in the nature protein.

Accordingly, one aspect of this invention pertains to isolated nucleic acids comprising nucleotide sequences encoding Der p VII or Der f VII, fragments thereof encoding peptides having at least one biological activity of Der p VII or Der f VII, and equivalents of such nucleic acids. The term nucleic acid as used herein is intended to include such fragments and equivalents. The term equivalent is intended to include nucleotide sequences encoding functionally equivalent Der p VII or Der f VII proteins or functionally equivalent peptides having an activity of Der p VII or Der f VII. As defined herein, a peptide having an activity of Der p VII or Der f VII has at least one biological activity of the Der p VII or Der f VII allergen. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants, and will also include sequences that differ from the nucleotide sequence encoding Der p VII or Der f VII shown in Figure 3A and 3B (SEQ ID NO: 1) or Figure 6A and 6B (SEQ ID NO: 6) due to the degeneracy of the genetic code. Equivalents will also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27°C below melting temperature (T_m) and about 1M salt) to the nucleotide sequence of Der p VII shown in Figure 3A and 3B (SEQ ID NO: 1) or Der f VII shown in Figure 6A and 6B (SEQ ID NO: 6).

Peptides referred to herein as having an activity of Der p VII or Der f VII or having a Der p VII or Der f VII activity are defined herein as peptides that have an amino acid sequence substantially corresponding to all or a portion of the amino acid sequence of Der p VII or Der f VII shown in Figure 3A and 3B (SEQ ID NO: 2) or Figure 6A and 6B (SEQ ID NO: 7), which peptide has at least one biological activity of Der p VII or Der f VII. For example, a peptide having an activity of Der p VII or Der f VII may have the ability to induce a response in Der p VII or Der f VII restricted T cells such as stimulation (e.g., T cell proliferation or cytokine secretion) or to induce T cell non-responsiveness. Alternatively, or additionally, a peptide having an activity of Der p VII or Der f VII may have the ability to bind (to be recognized by) immunoglobulin E (IgE) antibodies of dust mite-allergic subjects. Peptides which bind IgE are useful in methods of detecting allergic sensitivity to Der p VII or Der f VII in a subject. Peptides that do not bind IgE, or bind IgE to a lesser extent than a purified, native Der p VII or Der f VII protein binds IgE are particularly useful as therapeutic agents.

In one embodiment, the nucleic acid is a cDNA encoding a peptide having an activity of Der p VII or Der f VII. Preferably, the nucleic acid is a cDNA molecule comprising at least a portion of the nucleotide sequence encoding Der p VII shown in

Figure 3A and 3B (SEQ ID NO: 1) or Der f VII shown in Figure 6A and 6B (SEQ ID NO: 6). A preferred portion of the cDNA molecules of Figure 3A and 3B and Figure 6A and 6B includes the coding region of the molecule.

5 In another embodiment, the nucleic acid of the invention encodes a peptide having an activity of Der p VII or Der f VII and comprising an amino acid sequence shown in Figure 3A and 3B (SEQ ID NO:2) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 7) (Der f VII). Preferred nucleic acids encode a peptide having a Der p VII or Der f VII activity and having at least about 50% homology, more preferably at least about 60% homology and most preferably at least about 70% homology with the sequence shown in Figure 3A and 3B (SEQ ID NO: 1) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 6) (Der f VII).
10 Nucleic acids which encode peptides having a Der p VII or Der f VII activity and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence set forth in Figure 3A and 3B (SEQ ID NO: 2) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 7) (Der f VII) are also within the scope of the invention. Homology refers to sequence similarity between two peptides having an activity of Der p VII or Der f VII or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology
15 between sequences is a function of the number of matching or homologous positions shared by the sequences.

Another aspect of the invention provides a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid which encodes a peptide having all or a portion of an amino acid sequence shown in Figure 3A and 3B (SEQ ID NO: 2) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 7) (Der f VII). Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50° are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be
25 selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

Isolated nucleic acids encoding peptides having an activity of Der p VII or Der f VII, as described herein, and having a sequence which differs from the nucleotide
35 sequences shown in Figure 3A and 3B (SEQ ID NO: 1) and Figure 6A and 6B (SEQ ID

NO: 6) due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having an activity of Der p VII or Der f VII) but differ in sequence from the sequences of Figure 3A and 3B and Figure 6A and 6B due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the Der p VII or Der f VII protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequence of Der p VII or Der f VII will exist within the dust mite population. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-4% of the nucleotides) of the nucleic acids encoding peptides having an activity of Der p VII or Der f VII may exist among individual dust mites due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention. Furthermore, there may be one or more isoforms or related, cross-reacting family members of Der p VII or Der f VII. Such isoforms or family members are defined as proteins related in function and amino acid sequence to Der p VII or Der f VII, but encoded by genes at different loci.

Fragments of the nucleic acid encoding Der p VII or Der f VII are also within the scope of the invention. As used herein, a fragment of the nucleic acid encoding Der p VII or Der f VII refers to a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the entire amino acid sequence of Der p VII or Der f VII protein and which encodes a peptide having an activity of Der p VII or Der f VII (i.e., a peptide having at least one biological activity of the Der p VII or Der f VII allergen) as defined herein.

Preferred nucleic acid fragments encode peptides of at least about 7 amino acid residues in length, preferably about 13-40 amino acid residues in length, and more preferably about 16-30 amino acid residues in length. Nucleic acid fragments which encode peptides having a Der p VII activity of at least about 30 amino acid residues in length, at least about 40 amino acid residues in length, at least about 60 amino acid residues in length, at least about 80 amino acid residues in length, at least about 100 amino acid residues in length, at least about 140 residues in length, and at least about 190 residues in length or more are also within the scope of this invention. Nucleic acid fragments which encode peptides having a Der f VII activity of at least about 30 amino acid residues in length, at least about 40 amino acid residues in length, at least about 60 amino acid residues in length, at least about 80 amino acid residues in length, at least about 100 amino acid residues in length, at least about 140 residues in length, and at least about

200 amino acid residues in length or more are also within the scope of this invention. In general, expression of peptides in a transformed host cell is most advantageous where the desired peptide is greater than about 20 amino acids in length. Shorter peptides are typically more easily synthesized chemically.

5 Nucleic acid fragments within the scope of the invention include those capable of hybridizing under high or low stringency conditions with nucleic acids from other animal species for use in screening protocols to detect Der p VII or Der f VII or allergens that are cross-reactive with Der p VII or Der f VII. Generally, the nucleic acid encoding a peptide having an activity of Der p VII or Der f VII will be selected from the bases encoding the
10 mature protein, however, in some instances it may be desirable to select all or part of a peptide from the leader sequence portion of the nucleic acids of the invention. Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of recombinant peptides having an activity of Der p VII or Der f VII.

15 A nucleic acid encoding a peptide having an activity of Der p VII or Der f VII may be obtained from mRNA of the dust mite *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae*. It should also be possible to obtain nucleic acids encoding Der p VII or Der f VII from *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* genomic DNA. For example, the gene encoding Der p VII or Der f VII can be cloned
20 from either a cDNA or a genomic library in accordance with protocols herein described (see Examples 1 and 2). A cDNA encoding Der p VII or Der f VII can be obtained by isolating total mRNA from *Dermatophagoides pteronyssinus*. Double stranded cDNAs can then be prepared from the total mRNA. Subsequently, the cDNAs can be inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques.
25 Genes encoding Der p VII or Der f VII can also be cloned using established polymerase chain reaction techniques (see Examples 4 and 5) in accordance with the nucleotide sequence information provided by the invention. The nucleic acids of the invention can be DNA or RNA. A preferred nucleic acid is a cDNA encoding Der p VII or Der f VII having the sequence depicted in Figure 3A and 3B (SEQ ID NO:1) (Der p VII) or Figure
30 6A and 6B (SEQ ID NO: 6) (Der f VII).

This invention also provides expression vectors containing a nucleic acid encoding a peptide having an activity of Der p VII or Der f VII, operably linked to at least one regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is
35 linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the peptide having an activity of Der p VII or Der f VII. Accordingly, the term regulatory

sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. In one embodiment, the expression vector includes a DNA encoding a peptide having an activity of Der p VII or Der f VII. Such expression vectors can be used to transfect cells to thereby produce proteins or peptides, including fusion proteins or peptides encoded by nucleic acids as described herein.

This invention further pertains to a host cell transfected to express a peptide having an activity of Der p VII or Der f VII. The host cell may be any procaryotic or eucaryotic cell. For example, a peptide having an activity of Der p VII or Der f VII may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO). Other suitable host cells can be found in Goeddel, (1990) supra or known to those skilled in the art.

Expression in eucaryotic cells such as mammalian, yeast, or insect cells can lead to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of recombinant protein. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari. et al., (1987) Embo J., 6: 229 - 234), pMFa (Kurjan and Herskowitz, (1982) Cell, 30: 933 - 943), pJRY88 (Schultz et al., (1987) Gene, 54: 113 - 123), and pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983) Mol. Cell Biol., 3: 2156 - 2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) Virology, 170: 31 - 39). Generally COS cells (Gluzman, Y., (1981) Cell, 23: 175 - 182) are used in conjunction with such vectors as pCDM 8 (Aruffo, A. and Seed, B., (1987) Proc. Natl. Acad. Sci. USA, 84: 8573 - 8577) for transient amplification/expression in mammalian cells, while CHO (dhfr- Chinese Hamster Ovary) cells are used with vectors such as pMT2PC (Kaufman et al., (1987) EMBO J., 6: 187 - 195) for stable amplification/expression in mammalian cells. Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook et al., (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Expression in procaryotes is most often carried out in *E. coli* with either fusion or non-fusion inducible expression vectors. Fusion vectors usually add a number of NH₂ terminal amino acids to the expressed target gene. These NH₂ terminal amino acids often are referred to as a reporter group. Such reporter groups usually serve two purposes: 1) to increase the solubility of the target recombinant protein; and 2) to aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target recombinant protein to enable separation of the target recombinant protein from the reporter group subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann et al., (1988) Gene, 69: 301 - 315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology, 185, Academic Press, San Diego, California (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lac O fusion promoter mediated by coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant Der p VII or Der f VII expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., Gene Expression Technology: Methods in Enzymology, 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid encoding the Der p VII or Der f VII protein to be inserted into an expression vector so that the individual codons for each amino acid would be those preferentially utilized in highly expressed *E. coli* proteins (Wada et al., (1992) Nuc. Acids Res., 20: 2111 - 2118). Such alteration of nucleic acids of the invention can be carried out by standard DNA synthesis techniques.

The nucleic acids of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in

commercially available DNA synthesizers (See e.g., Itakura *et al.*, U.S. Patent No. 4,598,049; Caruthers *et al.*, U.S. Patent No. 4,458,066; and Itakura, U.S. Patent Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

This invention further pertains to methods of producing peptides that have an activity of Der p VII or Der f VII. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding a peptide having an activity of Der p VII or Der f VII can be cultured under appropriate conditions to allow expression of the peptide to occur. The peptide may be secreted and isolated from a mixture of cells and medium containing the peptide having an activity of Der p VII or Der f VII.

Alternatively, the peptide may be retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The peptide having an activity of Der p VII or Der f VII can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for a peptide having an activity of Der p VII or Der f VII.

Another aspect of the invention pertains to isolated peptides having an activity of Der p VII or Der f VII. A peptide having an activity of Der p VII or Der f VII has at least one biological activity of the Der p VII or Der f VII allergen. For example, a peptide having an activity of Der p VII or Der f VII may have the ability to induce a response in Der p VII or Der f VII specific T cells such as stimulation (T cell proliferation or cytokine secretion) or to induce T cell non-responsiveness. In one embodiment, a peptide having an activity of Der p VII or Der f VII stimulates T cells as evidenced by, for example, T cell proliferation or cytokine secretion. In another embodiment, peptides having a Der p VII or Der f VII activity induce T cell non-responsiveness in which T cells are unresponsive to a subsequent challenge with a Der p VII or Der f VII peptide following exposure to the peptide. In yet another embodiment, a peptide having a Der p VII or Der f VII activity has reduced IgE binding activity compared to purified, native Der p VII or Der f VII protein. A peptide having an activity of Der p VII or Der f VII may differ in amino acid sequence from the Der p VII or Der f VII sequence depicted in Figure 3A and 3B (SEQ ID NO:2) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 7) (Der f VII) but such differences result in a modified protein which functions in the same or similar manner as a native Der p VII or Der f VII protein or which has the same or similar characteristics of a native Der p VII or Der f VII protein. Various modifications of the Der p VII or Der f VII protein to

produce these and other functionally equivalent peptides are described in detail herein. The term peptide, as used herein, refers to full length proteins and polypeptides or peptide fragments thereof.

5 A peptide can be produced by modification of the amino acid sequence of the Der p VII or Der f VII protein shown in Figure 3A and 3B (SEQ ID NO: 2) (Der p VII) or Figure 6A and 6B (SEQ ID NO: 7) (Der f VII), such as a substitution, addition, or deletion of an amino acid residue which is not directly involved in the function of the protein. Peptides of the invention can be at least about 10 amino acid residues in length, preferably about 10-20 amino acid residues in length, and more preferably about 10-16 amino acid residues in length. Peptides having an activity of Der p VII or Der f VII and which are at least about 30 amino acid residues in length, at least about 40 amino acid residues in length, at least about 60 amino acid residues in length, at least about 80 amino acid residues in length, and at least about 100 amino acid residues in length are also included within the scope of this invention.

15 Another embodiment of the invention provides a substantially pure preparation of a peptide having an activity of Der p VII or Der f VII. Such a preparation is substantially free of proteins and peptides with which the peptide naturally occurs (i.e., other dust mite peptides), either in a cell or when secreted by a cell.

20 The term isolated as used herein refers to a nucleic acid or peptide that is substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Such proteins or peptides are also characterized as being free of all other dust mite proteins. Accordingly, an isolated peptide having an activity of Der p VII or Der f VII is produced recombinantly or synthetically and is substantially free of cellular material and culture medium or substantially free of chemical precursors or other chemicals and is substantially free of all other dust mite proteins. An isolated nucleic acid is also free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the organism from which the nucleic acid is derived.

25 Peptides having an activity of Der p VII or Der f VII can be obtained, for example, by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid of Der p VII or Der f VII encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, the Der p VII or Der f VII protein may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify

those peptides having a Der p VII or Der f VII activity (i.e., the ability to induce a T cell response such as stimulation (proliferation, cytokine secretion), nonresponsiveness, and/or has reduced IgE binding activity).

In one embodiment, peptides having an activity of Der p VII or Der f VII can be identified by the ability of the peptide to stimulate T cells or to induce T cell non-responsiveness. Peptides which stimulate T cells, as determined by, for example, T cell proliferation or cytokine secretion are defined herein as comprising at least one T cell epitope. T cell epitopes are believed to be involved in initiation and perpetuation of the immune response to the protein allergen which is responsible for the clinical symptoms of allergy. These T cell epitopes are thought to trigger early events at the level of the T helper cell by binding to an appropriate HLA molecule on the surface of an antigen presenting cell, thereby stimulating the T cell subpopulation with the relevant T cell receptor for the epitope. These events lead to T cell proliferation, lymphokine secretion, local inflammatory reactions, recruitment of additional immune cells to the site of antigen/T cell interaction, and activation of the B cell cascade, leading to the production of antibodies. One isotype of these antibodies, IgE, is fundamentally important to the development of allergic symptoms and its production is influenced early in the cascade of events at the level of the T helper cell, by the nature of the lymphokines secreted. A T cell epitope is the basic element, or smallest unit of recognition by a T cell receptor, where the epitope comprises amino acids essential to receptor recognition. Amino acid sequences which mimic those of the T cell epitopes and which modify the allergic response to protein allergens are within the scope of this invention.

Screening peptides for those which retain a Der p VII or Der f VII activity as described herein can be accomplished using one or more of several different assays. For example, *in vitro*, Der p VII or Der f VII T cell stimulatory activity is assayed by contacting a peptide known or suspected of having a Der p VII or Der f VII activity with an antigen presenting cell which presents appropriate MHC molecules in a T cell culture. Presentation of a peptide having a Der p VII or Der f VII activity in association with appropriate MHC molecules to T cells in conjunction with the necessary costimulation has the effect of transmitting a signal to the T cell that induces the production of increased levels of cytokines, particularly of interleukin-2 and interleukin-4. The culture supernatant can be obtained and assayed for interleukin-2 or other known cytokines. For example, any one of several conventional assays for interleukin-2 can be employed, such as the assay described in Proc. Natl. Acad. Sci USA, 86: 1333 (1989) the pertinent portions of which are incorporated herein by reference. A kit for an assay for the production of interferon is also available from Genzyme Corporation (Cambridge, MA).

Alternatively, a common assay for T cell proliferation entails measuring tritiated thymidine incorporation. The proliferation of T cells can be measured *in vitro* by determining the amount of ³H-labeled thymidine incorporated into the replicating DNA of cultured cells. Therefore, the rate of DNA synthesis and, in turn, the rate of cell division can be quantified.

In one embodiment, peptides which have Der p VII or Der f VII T cell stimulating activity (i.e., the peptide comprises at least one T cell epitope) can be identified using an algorithm which predicts the presence of T cell epitopes in a protein sequence, such as the algorithm described by Hill et al., Journal of Immunology, 147:189-197 (1991). The algorithm of Hill et al. predicts the location of T cell epitopes in a protein by the presence of certain patterns within the sequence which are likely to bind MHC and therefore may contain T cell epitopes.

In order to determine precise T cell epitopes by, for example, fine mapping techniques, a peptide having Der p VII or Der f VII T cell stimulating activity and thus comprising at least one T cell epitope as determined by T cell biology techniques is modified by addition or deletion of amino acid residues at either the amino or carboxy terminus of the peptide and tested to determine a change in T cell reactivity to the modified peptide. Following this technique, peptides are selected and produced recombinantly or synthetically. Peptides are selected based on various factors, including the strength of the T cell response to the peptide (e.g., stimulation index), the frequency of the T cell response to the peptide in a population of individuals sensitive to dust mite allergens, and the potential cross-reactivity of the peptide with other dust mite allergens. The physical and chemical properties of these selected peptides (e.g., solubility, stability) are examined to determine whether the peptides are suitable for use in therapeutic compositions or whether the peptides require modification as described herein. The ability of the selected peptides or selected modified peptides to stimulate human T cells (e.g., induce proliferation, lymphokine secretion) is then determined as described herein.

In another embodiment, a peptide having a Der p VII or Der f VII activity is screened for the ability to induce T cell non-responsiveness. The ability of a peptide known to stimulate T cells (as determined by one or more of the above described assays), to inhibit or completely block the activity of purified native Der p VII or Der f VII or portion thereof and induce a state of non-responsiveness can be determined using subsequent attempts at stimulation of the T cells with antigen presenting cells that present native Der p VII or Der f VII or peptide having a Der p VII or Der f VII activity following exposure to the peptide, having a Der p VII or Der f VII activity. If the T cells are unresponsive to the subsequent activation attempts, as determined by interleukin-2

synthesis and/or T cell proliferation, a state of non-responsiveness has been induced. See, e.g., Gimmi et al., (1993) Proc. Natl. Acad. Sci USA, 90: 6586 - 6590; and Schwartz (1990) Science, 248: 1349 - 1356, for assay systems that can be used as the basis for an assay in accordance with the present invention.

5 In yet another embodiment, peptides having a Der p VII or Der f VII activity are identified by IgE binding activity. For therapeutic purposes, peptides of the invention preferably do not bind IgE specific for a dust mite allergen, or bind such IgE to a substantially lesser extent (e.g., at least 100-fold, less, more preferred at least 1000-fold less) than the corresponding purified native dust mite allergen binds such IgE. Reduced
10 IgE binding activity refers to IgE binding activity that is less than that of purified native Der p VII or Der f VII protein. If a peptide having a Der p VII or Der f VII activity is to be used as a diagnostic reagent, it is not necessary that the peptide have reduced IgE binding activity compared to the native Der p VII or Der f VII allergen. IgE binding activity of peptides can be determined by, for example, an enzyme-linked immunosorbent
15 assay (ELISA) using, for example, sera obtained from a subject, (i.e., an allergic subject) that has been previously exposed to the native Der p VII or Der f VII allergen. Briefly, the peptide suspected of having a Der p VII or Der f VII activity is coated onto wells of a microtiter plate. After washing and blocking the wells, antibody solution consisting of the plasma of an allergic subject who has been exposed to a peptide suspected of having a Der
20 p VII or Der f VII activity is incubated in the wells. The plasma is generally depleted of IgG before incubation. A labeled secondary antibody is added to the wells and incubated. The amount of IgE binding is then quantified and compared to the amount of IgE bound by a purified, native Der p VII or Der f VII protein. Alternatively, the IgE binding activity of a peptide can be determined by Western blot analysis. For example, a peptide suspected of
25 having a Der p VII or Der f VII activity is run on a polyacrylamide gel using SDS-PAGE. The peptide is then transferred to nitrocellulose and subsequently incubated with sera from an allergic subject. After incubation with a labeled secondary antibody, the amount of IgE bound is then determined and quantified.

Another assay which can be used to determine the IgE binding activity of a peptide
30 is a competition ELISA assay. Briefly, an IgE antibody pool is generated by combining plasma from dust mite allergic subjects that have been shown by direct ELISA to have IgE reactive with native Der p VII or Der f VII. This pool is used in ELISA competition assays to compare IgE binding of native Der p VII or Der f VII and a peptide suspected of having a Der p VII or Der f VII activity. IgE binding for the native Der p VII or Der f
35 VII protein and a peptide suspected of having a Der p VII or Der f VII activity is determined and quantified.

If a peptide having an activity of Der p VII or Der f VII binds IgE, and is to be used as a therapeutic agent, it is preferable that such binding does not result in the release of mediators (e.g., histamines) from mast cells or basophils. To determine whether a peptide which binds IgE results in the release of mediators, a histamine release assay can be performed using standard reagents and protocols obtained, for example, from Amac, Inc. (Westbrook, ME). Briefly, a buffered solution of a peptide suspected of having a Der p VII or Der f VII activity is combined with an equal volume of whole heparinized blood from an allergic subject. After mixing and incubation, the cells are pelleted and the supernatants are processed and analyzed using a radioimmunoassay to determine the amount of histamine released.

Peptides having an activity of Der p VII or Der f VII which are to be used as therapeutic agents are preferably tested in mammalian models of dust mite atopy, such as the mouse model disclosed in Tamura et al., (1986) Microbiol. Immunol., 30: 883 - 896, or in U.S. Patent 4,939,239, or in the primate model disclosed in Chiba et al., (1990) Int. Arch. Allergy Immunol., 93: 83 - 88. Initial screening for IgE binding to a peptide having an activity of Der p VII or Der f VII may be performed by scratch tests or intradermal skin tests on laboratory animals or human volunteers, or in *in vitro* systems such as RAST, RAST inhibition, ELISA assay, RIA (radioimmunoassay), or a histamine release assay, as described above.

It is possible to modify the structure of a peptide having an activity of Der p VII or Der f VII for such purposes as increasing solubility, enhancing therapeutic or prophylactic efficacy, or stability (e.g., shelf life *ex vivo* and resistance to proteolytic degradation *in vivo*). Such modified peptides are considered functional equivalents of peptides having an activity of Der p VII or Der f VII as defined herein. A modified peptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition, to modify immunogenicity and/or reduce allergenicity, or to which a component has been added for the same purpose.

For example, a peptide having an activity of Der p VII or Der f VII can be modified so that it maintains the ability to induce T cell non-responsiveness and bind MHC proteins without the ability to induce a strong proliferative response or possibly, any proliferative response when administered in immunogenic form. In this instance, critical binding residues for T cell receptor function can be determined using known techniques (e.g., substitution of each residue and determination of the presence or absence of T cell reactivity). Those residues shown to be essential to interact with the T cell receptor can be modified by replacing the essential amino acid with another, preferably similar amino acid residue (a conservative substitution) whose presence is shown to enhance, diminish but not

eliminate, or not affect T cell reactivity. In addition, those amino acid residues which are not essential for T cell receptor interaction can be modified by being replaced by another amino acid whose incorporation may enhance, diminish but not eliminate, or not affect T cell reactivity, but does not eliminate binding to relevant MHC.

5 Additionally, a peptide having an activity of Der p VII or Der f VII can be modified by replacing an amino acid shown to be essential to interact with the MHC protein complex with another, preferably similar amino acid residue (conservative substitution) whose presence is shown to enhance, diminish but not eliminate, or not affect T cell activity. In addition, amino acid residues which are not essential for interaction with
10 the MHC protein complex but which still bind the MHC protein complex can be modified by being replaced by another amino acid whose incorporation may enhance, not affect, or diminish but not eliminate T cell reactivity. Preferred amino acid substitutions for non-essential amino acids include, but are not limited to substitutions with alanine, glutamic acid, or a methyl amino acid.

15 Another example of modification of a peptide having an activity of Der p VII or Der f VII is substitution of cysteine residues preferably with alanine, serine, threonine, leucine or glutamic acid residues to minimize dimerization via disulfide linkages. In addition, amino acid side chains of fragments of the protein of the invention can be chemically modified. Another modification is cyclization of the peptide.

20 In order to enhance stability and/or reactivity, a peptide having an activity of Der p VII or Der f VII can be modified to incorporate one or more polymorphisms in the amino acid sequence of the protein allergen resulting from any natural allelic variation.

Additionally, D-amino acids, non-natural amino acids, or non-amino acid analogs can be substituted or added to produce a modified protein within the scope of this invention.

25 Furthermore, a peptide having an activity of Der p VII or Der f VII can be modified using polyethylene glycol (PEG) according to the method of A. Sehon and co-workers (Wie et al., *supra*) to produce a protein conjugated with PEG. In addition, PEG can be added during chemical synthesis of the protein. Other modifications of a peptide having an activity of Can f Ior Der f VII include reduction/alkylation (Tarr, *Methods of Protein*
30 *Microcharacterization*, J. E. Silver ed., Humana Press, Clifton NJ 155-194 (1986)); acylation (Tarr, *supra*); chemical coupling to an appropriate carrier (Mishell and Shiigi, eds, *Selected Methods in Cellular Immunology*, WH Freeman, San Francisco, CA (1980), U.S. Patent 4,939,239; or mild formalin treatment (Marsh, (1971) *Int. Arch. of Allergy and Appl. Immunol.*, 41: 199 - 215).

To facilitate purification and potentially increase solubility of a peptide having an activity of Der p VII or Der f VII, it is possible to add an amino acid fusion moiety to the peptide backbone. For example, hexa-histidine can be added to the protein for purification by immobilized metal ion affinity chromatography (Hochuli, E. *et al.*, (1988)

5 Bio/Technology, 6: 1321 - 1325). In addition, to facilitate isolation of peptides free of irrelevant sequences, specific endoprotease cleavage sites can be introduced between the sequences of the fusion moiety and the peptide. In order to successfully desensitize a subject to Der p VII or Der f VII protein or related allergen, it may be necessary to increase the solubility of the protein by adding functional groups to the protein, or by
10 omitting hydrophobic regions of the protein. Functional groups, such as charged amino acids and charged amino acid pairs are suitable for increasing solubility when added to the amino or carboxy terminus of the peptide.

To potentially aid proper antigen processing of T cell epitopes within Der p VII or Der f VII, canonical protease sensitive sites can be engineered between regions, each
15 comprising at least one T cell epitope via recombinant or synthetic methods. For example, charged amino acid pairs, such as KK or RR, can be introduced between regions within a protein or fragment during recombinant construction thereof. The resulting peptide can be rendered sensitive to cleavage by cathepsin and/or other trypsin-like enzymes which would generate portions of the protein containing one or more T cell epitopes. In addition, such
20 charged amino acid residues can result in an increase in the solubility of the peptide.

Site-directed mutagenesis of a nucleic acid encoding a peptide having an activity of Der p VII or Der f VII can be used to modify the structure of the peptide by methods known in the art. Such methods may, among others, include polymerase chain reaction (PCR) with oligonucleotide primers bearing one or more mutations (Ho *et al.*, (1989)
25 Gene, 77: 51 - 59) or total synthesis of mutated genes (Hostomsky, Z. *et al.*, (1989) Biochem. Biophys. Res. Comm., 161: 1056 - 1063). To enhance recombinant protein expression, the aforementioned methods can be applied to change the codons present in the cDNA sequence of the invention to those preferentially utilized by the host cell in which the recombinant protein is being expressed (Wada *et al.*, *supra*).

30 Another aspect of the invention pertains to an antibody specifically reactive with a peptide having an activity of Der p VII or Der f VII. The antibodies of this invention can be used to standardize allergen extracts or to isolate the naturally-occurring or native form of Der p VII or Der f VII. For example, by using peptides having an activity of Der p VII or Der f VII based on the cDNA sequence of Der p VII or Der f VII, anti-protein/anti-peptide antisera or monoclonal antibodies can be made using standard methods. A
35 mammal such as a mouse, a hamster or rabbit can be immunized with an immunogenic

form of the peptide (e.g., Der p VII or Der f VII protein or an antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. A peptide having an activity of Der p VII or Der f VII can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization, anti-Der p VII or anti-Der f VII antisera can be obtained and, if desired, polyclonal anti-Der p VII or anti-Der f VII antibodies isolated from the serum. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, for example the hybridoma technique originally developed by Kohler and Milstein, (1975) Nature, 256: 495 - 497) as well as other techniques such as the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a peptide having an activity of Der p VII or Der f VII and the monoclonal antibodies isolated.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with the peptide having an activity of Der p VII or Der f VII. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having an anti-Der p VII or anti-Der f VII portion.

Another aspect of this invention provides T cell clones and soluble T cell receptors specifically reactive with a peptide having an activity of Der p VII or Der f VII. Monoclonal T cell populations (i.e., T cells genetically identical to one another and expressing identical T cell receptors) can be derived from an individual sensitive to Der p VII or Der f VII, followed by repetitive *in vitro* stimulation with a Der p VII or Der f VII protein or peptide having an activity of Der p VII or Der f VII in the presence of MHC-matched antigen-presenting cells. Single Der p VII or Der f VII MHC responsive cells can then be cloned by limiting dilution and permanent lines expanded and maintained by

periodic *in vitro* restimulation. Alternatively, Der p VII or Der f VII specific T-T hybridomas can be produced by a technique similar to B cell hybridoma production. For example, a mammal, such as a mouse, is immunized with a peptide having an activity of Der p VII or Der f VII, T cells are then purified and fused with an autonomously growing T cell tumor line. From the resulting hybridomas, cells responding to a peptide having an activity of Der p VII or Der f VII are selected and cloned. Procedures from propagating monoclonal T cell populations are described in Cellular and Molecular Immunology (Abul K. Abbas et al. ed.), W.B. Saunders Company, Philadelphia, PA (1991) page 139.

Soluble T cell receptors specifically reactive with a peptide having an activity of Der p VII or Der f VII can be obtained by immunoprecipitation using an antibody against the T cell receptor as described in Immunology: A Synthesis (Second Edition), Edward S. Golub et al., ed., Sinauer Associates, Inc., Sunderland, MA (1991) pages 366-269.

T cell clones specifically reactive with a peptide having an activity of Der p VII or Der f VII can be used to isolate and molecularly clone the gene encoding the relevant T cell receptor. In addition, a soluble T cell receptor specifically reactive with a peptide having an activity of Der p VII or Der f VII can be used to interfere with or inhibit antigen-dependent activation of the relevant T cell subpopulation, for example, by administration to an individual sensitive to Der p VII or Der f VII. Antibodies specifically reactive with such a T cell receptor can be produced according to the techniques described herein. Such antibodies can be used to block or interfere with the T cell interaction with peptides presented by MHC.

Exposure of allergic subjects to peptides having an activity of Der p VII or Der f VII and which have T cell stimulating activity, may cause the appropriate T cell subpopulations to become non-responsive to the respective protein allergen (e.g., fail to stimulate an immune response upon such exposure). In addition, such administration may modify the lymphokine secretion profile as compared with exposure to the naturally-occurring protein allergen or portion thereof (e.g., result in a decrease of IL-4 and/or an increase in IL-2). Furthermore, exposure to peptides having an activity of Der p VII or Der f VII which have T cell stimulating activity may influence T cell subpopulations which normally participate in the response to the allergen such that these T cells are drawn away from the site(s) of normal exposure to the allergen (e.g., nasal mucosa, skin, and lung) towards the site(s) of therapeutic administration of the protein or fragment derived therefrom. This redistribution of T cell subpopulations may ameliorate or reduce the ability of an individual's immune system to stimulate the usual immune response at the site of normal exposure to the allergen, resulting in a diminution in allergic symptoms.

A peptide having an activity of Der p VII or Der f VII when administered to a subject sensitive to dust mite allergens is capable of modifying the B cell response, T cell response, or both the B cell and the T cell response of the subject to the allergen. As used herein, modification of the allergic response of a subject to a dust mite allergen can be defined as non-responsiveness or diminution in symptoms to the allergen, as determined by standard clinical procedures (See e.g., Varney et al., (1990) British Medical Journal, 302: 265 - 269), including diminution in dust mite induced asthmatic symptoms. As referred to herein, a diminution in symptoms includes any reduction in the allergic response of a subject to the allergen following a treatment regimen with a peptide of the invention. This diminution in symptoms may be determined subjectively (e.g., the patient feels more comfortable upon exposure to the allergen), or clinically, such as with a standard skin test.

Peptides or antibodies of the present invention can also be used for detecting and diagnosing sensitivity to Der p VII or Der f VII. For example, this can be done *in vitro* by combining blood or blood products obtained from a subject to be assessed for sensitivity with peptide having an activity of Der p VII or Der f VII, under conditions appropriate for binding of components in the blood (e.g., antibodies, T cells, B cells) with the peptide(s) and determining the extent to which such binding occurs. Other diagnostic methods for allergic diseases which the peptides or antibodies of the present invention can be used include radio-allergosorbent test (RAST), paper radioimmunosorbent test (PRIST), enzyme linked immunosorbent assay (ELISA), radioimmunoassays (RIA), immuno-radiometric assays (IRMA), luminescence immunoassays (LIA), histamine release assays and IgE immunoblots.

The present invention further provides methods of detecting and treating sensitivity in a subject to Der p VII or Der f VII. The presence in subjects of IgE specific for Der p VII or Der f VII and the ability of T cells of the subjects to respond to T cell epitopes of Der p VII or Der f VII can be determined by administering to the subject an Immediate Type Hypersensitivity test and/or a Delayed Type Hypersensitivity test (See e.g., Immunology (1985) Roitt, I.M., Brostoff, J., Male, D.K. (eds), C.V. Mosby Co., Gower Medical Publishing, London, NY, pp. 19.2-19.18; pp.22.1-22.10) utilizing a peptide having an activity of Der p VII or Der f VII, or a modified form of a peptide having an activity of Der p VII or Der f VII, each of which binds IgE specific for the allergen. The same subjects are administered a Delayed Type Hypersensitivity test prior to, simultaneously with, or subsequent to administration of the Immediate Type Hypersensitivity test. Of course, if the Immediate Type Hypersensitivity test is administered prior to the Delayed Type Hypersensitivity test, the Delayed Type Hypersensitivity test would be given to those subjects exhibiting a specific Immediate Type

Hypersensitivity reaction. The Delayed Type Hypersensitivity test utilizes a peptide having an activity of Der p VII or Der f VII which has human T cell stimulating activity and which does not bind IgE specific for the allergen in a substantial percentage of the population of subjects sensitive to the allergen (e.g., at least about 75%). Those subjects found to have both a specific Immediate type Hypersensitivity reaction and a specific Delayed Type Hypersensitivity reaction are administered an amount of a composition suitable for pharmaceutical administration. The composition comprises the peptide having an activity of Der p VII or Der f VII as used in the Delayed Type Hypersensitivity test and a pharmaceutically acceptable carrier or diluent.

A peptide having an activity of Der p VII or Der f VII can be used in methods of diagnosing, treating, or preventing allergic reactions to a dust mite allergen or a cross-reactive protein allergen. Thus, the present invention provides compositions suitable for *in vitro* use and pharmaceutical administration comprising an amount of at least one peptide having an activity of Der p VII or Der f VII. Pharmaceutical compositions typically will be formulated with a pharmaceutically acceptable carrier.

Where a composition according to the invention is intended for administration to a subject to be desensitized, such administration can be carried out using known procedures, at dosages and for periods of time effective to reduce sensitivity (i.e., reduce the allergic response) of the subject to a dust mite allergen. The term subject is intended to include living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. An amount of at least one peptide having an activity of Der p VII or Der f VII necessary to achieve a therapeutic effect may vary according to factors such as the degree of sensitivity of the subject to dust mite, the age, sex, and weight of the subject, and the ability of a peptide having an activity of Der p VII or Der f VII to elicit an antigenic response in the subject. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound (i.e., a peptide having an activity of Der p VII or Der f VII) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

To administer a peptide having an activity of Der p VII or Der f VII by other than parenteral administration, it may be necessary to coat the peptide with, or co-administer the peptide with, a material to prevent its inactivation. For example, a peptide having an activity of Der p VII or Der f VII may be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan et al., (1984) J. Neuroimmunol., 7: 27). For purposes of inducing T cell nonresponsiveness, the composition is preferably administered in non-immunogenic form, e.g., one that does not contain adjuvant.

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars,

polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

5 Sterile injectable solutions can be prepared by incorporating active compound (i.e., a peptide having an activity of Der p VII or Der f VII) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion
10 medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (i.e., at least one peptide having an activity of Der p VII or Der f VII) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

15 When the peptide having an activity of Der p VII or Der f VII is suitably protected, as described above, the peptide may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The peptide and other ingredients may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. For oral therapeutic administration, the active
20 compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage
25 will be obtained.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or
30 agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein
35 refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to

10024955-121901

produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in subjects.

The present invention also provides a composition comprising at least two peptides having an activity of Der p VII or Der f VII (e.g., a physical mixture of at least two peptides), each having T cell stimulating activity. For example, at least two peptides each having an activity of Der p VII can be combined or at least two peptides each having an activity of Der f VII can be combined, or at least one peptide having an activity of Der p VII and at least one peptide having an activity of Der f VII can be combined and administered. Alternatively, a peptide having at least two regions, each having T cell stimulating activity (i.e., each region comprising at least one T cell epitope) can be administered to an allergic subject. Such a peptide can have at least two regions derived from the same allergen, Der p VII or Der f VII, or a combination of Der p VII and Der f VII. A composition of two peptides or a peptide having at least two regions can be administered to a subject in the form of a composition with a pharmaceutically acceptable carrier as hereinbefore described. An amount of one or more of such compositions can be administered simultaneously or sequentially to a subject sensitive to a dust mite allergen to treat such sensitivity. Such compositions may be useful for the manufacture of a medicament for treating sensitivity to house dust mites in an individual.

The cDNA (or the mRNA which served as a template during reverse transcription) encoding a peptide having an activity of Der p VII or Der f VII can be used to identify similar nucleic acid sequences in any variety or type of animal and, thus, to molecularly clone genes which have sufficient sequence homology to hybridize to the cDNA encoding a peptide having an activity of Der p VII or Der f VII. Thus, the present invention includes not only peptides having an activity of Der p VII or Der f VII, but also other proteins which may be allergens encoded by DNA which hybridizes to DNA of the present invention.

Isolated peptides that are immunologically related to Der p VII or Der f VII, such as by antibody cross-reactivity or T cell cross-reactivity, other than those already identified, are within the scope of the invention. Such peptides bind antibodies specific for the protein and peptides of the invention, or stimulate T cells specific for the protein and peptides of this invention.

1004955-1200T

1 A peptide having an activity of Der p VII or Der f VII (i.e., Der p VII or Der f VII
2 produced recombinantly or by chemical synthesis) is free of all other dust mite proteins
3 and, thus, is useful in the standardization of allergen extracts which are key reagents for
4 the diagnosis and treatment of dust mite hypersensitivity. In addition, such a peptide is of
5 a consistent, well-defined composition and biological activity for use in preparations which
6 can be administered for therapeutic purposes (e.g., to modify the allergic response of a
7 subject sensitive to dust mite). Such peptides can also be used to study the mechanism of
8 immunotherapy of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* allergy
9 and to design modified derivatives or analogs useful in immunotherapy.

10 Work by others has shown that high doses of allergen extracts generally produce
11 the best results during immunotherapy (i.e., best symptom relief). However, many
12 subjects are unable to tolerate large doses of such extracts due to systemic reactions elicited
13 by the allergens and other components within these preparations. A peptide having an
14 activity of Der p VII or Der f VII according to the invention has the advantage of being
15 free of all other dust mite proteins, and thus are safer and more suitable for therapeutic
16 uses.

17 It is now also possible to design an agent or a drug capable of blocking or inhibiting
18 the ability of a dust mite allergen to induce an allergic reaction in sensitive subjects. Such
19 agents could be designed, for example, in such a manner that they would bind to relevant
20 anti-Der p VII or anti-Der f VII IgE molecules, thus preventing IgE-allergen binding, and
21 subsequent mast cell/basophil degranulation. Alternatively, such agents could bind to
22 cellular components of the immune system, resulting in suppression or desensitization of
23 the allergic responses to dust mite allergens. A non-restrictive example of this is the use of
24 peptides including B or T cell epitopes of Der p VII or Der f VII, or modifications thereof,
25 based on the cDNA protein structure of Der p VII or Der f VII to suppress the allergic
26 response to a dust mite allergen. This could be carried out by defining the structures of
27 fragments encoding B and T cell epitopes which affect B and T cell function in *in vitro*
28 studies with blood components from subjects sensitive to dust mite.

29 The invention is further illustrated by the following examples which should not be
30 construed as further limiting the subject invention. The contents of all references and
31 published patent applications cited throughout this application are hereby incorporated by
32 reference.

Example 1 - Isolation of clone HD6 from a λ gt11 cDNA library

A λ gt11 cDNA library was prepared from live adult *Dermatophagoides pteronyssinus* purchased from the Commonwealth Serum Laboratories, Parkville, Australia (Thomas, W., et al., Int Arch Allergy Appl Immunol (1988) 85:127-9). The library was prepared according to Chua et al. (J. Exp. Med. (1988) 167:175-182) based upon the method of Young and Davis (5 Proc. Natl. Acad. Sci. USA (1983) 80:1194-1198) and Gubler and Hoffman (Gene (1983) 25:263-299). Polyadenylated mRNA was isolated from a *D. pteronyssinus* culture and cDNA synthesized by the RNaseH method (Gubler and Hoffman, *supra*) using a kit (Amersham International, Bucks). After the addition of EcoRI linkers the cDNA was ligated into λ gt11 10 and plated in *E. coli* Y1090 (r-) (Promega Biotec, Madison, Wisconsin) to produce a library of 5×10^5 recombinants.

Allergic serum was used to probe the λ gt11 library. IgE plaque immunoassays were conducted by a standard procedure (Chua, K.Y., et al., Int Arch Allergy Appl Immunol (1990) 91:118-23) using 20 000 pfu on 14.5 cm petri dishes. Briefly, an overnight culture of 15 *E. coli* Y1090 (Huynh, T.V. et al. Constructing and Screening cDNA Libraries in gt10 and gt11 in: A Practical Approach, Oxford IRL Press, 1986, pp 48-78) was diluted 1/50 in L broth and incubated at 37°C to an OD₆₅₀ of 0.6. The bacteria were pelleted and resuspended in 400 μ l for every 50 ml of broth. For 14.5-cm Petri dishes, 300 μ l of Y1090 were incubated with 10^4 pfu phage for 30 minutes at room temperature and then plated on LB agar in 9 ml of 20 0.7% agar overlay and incubated for 3 hours at 42°C (when plaques usually become visible). At this time a nitrocellulose filter, which had been saturated with 10 mM isopropyl β -D-thiogalactoside and dried, was placed on top of the lawn. The incubation continued overnight at 37°C. The filter was then removed and washed in 0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.05% Tween 20 v/v, pH 8, (TNT) buffer with gentle rocking for 20 minutes. The 25 filter was then incubated with sera from mite allergic children for 2 hours at room temperature with rocking and then washed three times for 30 minute periods with TNT. The sera used was first diluted 50:50 with an *E. coli* extract (Huynh et al., *supra*), incubated overnight then clarified by centrifugation (3,000 g 10 minutes). Non-fat milk and sodium azide were added to 5 and 0.02%, respectively. To develop the IgE reactivity the filter was 30 rocked in a solution of 125 I-labelled anti-IgE for 2 hours at room temperature followed by three 30-minute washes with TNT. The anti-IgE was a mouse monoclonal 2.1.5 (available from Silenus Laboratories Pty. Ltd., Hawthorn, Victoria) and was used at 30 ng/ml coupled with 10^5 dpm/ng 125 I in TNT (Stewart, G.A., et al. Int. Arch. Allergy Appl. Immunol. (1988) 86:9-18). It was labelled by the chloramine T method. The filter was 35 autoradiographed with intensifying screens, usually for 48 hours at -70°C.

A λ gt11 derived clone HD6 from the *D. pteronyssinus* cDNA library was plaque purified (see Maniati *et al.* Molecular Cloning: A Laboratory Manual, (1982) Cold Spring Harbor) because it showed high IgE binding activity to a mite allergic serum (obtained from a child attending the allergy clinic at the Taiwan University Hospital, Taipei, R.O.C.) by the plaque radioimmune assay described above. To determine the number of sera with IgE binding for this clone, the λ gt11-HD6 was plated at 1 000 pfu on a 90 cm petri dish and a nitrocellulose lift prepared for an immunoassay as outlined in Young and Davis (1983) *supra*, with modifications as detailed in Chua *et al.* Int. Arch. Allergy Appl. Immunol. (1990) 91:118-123. The filter was cut into segments and IgE immunoassays performed with 20 individual sera obtained from the Royal Children's Hospital Melbourne (Dr. D. Hill) (Fig. 1). Strong reactivity was found with 6 sera and in another series with 8/18. A hyper IgE serum tested at 1 000 IU/ml did not show binding, nor did a serum from a child allergic to only rye grass (see bottom two segments in right-hand column of Fig. 1).

To estimate the size of the IgE binding molecule encoded by the phage, DNA from purified clones was isolated by a polyethyleneglycol precipitation procedure (Chua, K.Y. *et al.* J. Exp. Med. (1988) 167:175-182) and the 812 bp DNA insert found in the λ gt11-HD6 was released by EcoRI digestion (Toyobo, Osaka, Japan) and subcloned into the same site in the glutathione-S-transferase fusion vector pGEX-1 (Smith, D.B. *et al.*, Gene (1988) 67:31-40) and used to transform *E. coli* TG-1. The protein expressed by this construct was isolated from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized a glutathione (as described in Smith *et al.* Gene (1988) 67:31-40). The fusion protein was then examined by Western blotting. For Western Blot Analysis, proteins were transferred to nitrocellulose (Bio-Rad transblot) by the protocol of Burnette (Burnette, W.N., Anal Biochem (1981) 112:195-203) and immunoassays were performed as for the plaque radioimmune assays with allergic sera and 125 I-anti IgE or with rabbit antibodies and 125 I-protein A as described in Greene, W.K., *et al.*, Int Arch Allergy Appl Immunol (1990) 92:30-8.

Expression in pGEX-1 resulted in a protein(s) which migrated as a doublet with a Mr of 53-55 K and reacted by Western blotting with rabbit anti-house dust mite serum (Fig. 2, lane 1). Two allergic sera reacted with this doublet (Fig. 2, lanes 3 and 5) but not to a hyper IgE serum at 1000 IU/ml (Fig. 2, lane 4) or normal rabbit serum (Fig. 2, lane 2). The IgE binding protein, allowing for the contribution of the 27 K glutathione transferase would therefore be about Mr 27. This, as will be described below, contains residues from the leader sequence and those from the 5' untranslated region.

Example 2 - DNA sequence analysis of clone HD6

The 812 bp insert of clone HD6 was cloned into the M13 vectors mp18 and mp19 (see Messing Methods Enzymology (1983) 101:20) for sequencing performed in both directions using -40, universal and internal primers (Messing, *supra*). Dideoxynucleotide sequencing (Sambrook, J., et al. Molecular Cloning. A Laboratory Manual. 2nd Edition. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989) was performed using a Sequenase 2.0 kit (IBI, New Haven, USA) with ^{32}P -dATP and a Biorad Sequi-gen electrophoresis apparatus. Following sequence analysis with the universal and internal primers, three primers based on the cDNA sequence of Der p VII were produced and sequencing conducted. The sequences of the primers are as follows: (1) GATCCAATTCACTATGAT (bases 119-136 in Fig. 3, SEQ ID NO:3); (2) GGTGAATTAGACATGCG (bases 272-288 in Fig. 3, SEQ ID NO:4); and (3) TCAATTTTGGATCCAATTTTCGCT (bases 584-607, SEQ ID NO:5).

The DNA insert was found to have 812 bases with an open reading frame beginning at the 5' end, consistent with its expression as a fusion from λ gt11 and pGEX-1, and ending at a stop codon TAG (713-715) (Fig. 3). The sequence of the translated protein appeared to begin at the adjacent initiation ATGs at nucleotides 68-70 and 71-73. This is followed by nucleotides encoding a typical, predominantly hydrophobic, leader sequence (Von Hiejne, G. J. Mol. Biol. (1985) 184:99-105) predicted to be 17 residues long, and then a sequence encoding a further 198 residues ending at the TAG codon at nucleotides 713-715. This reading frame was confirmed by using PCR (as described in Saiki et al. Science (1988) 239:487-491) to clone DNA encoding an antigenic product of the correct Mr in pGEX starting at the predicted N-terminal Asp encoded by nucleotide 119-121. The fusion protein from this construct was produced at far higher yields than the fusion which contained the leader peptide (pGEX-1). The 3' untranslated region contained a polyadenylation signal AATAAA at 765-770 (underlined in Fig. 3) and a polyA tail. A potential N-glycosylation site, Asn Ala Thr, is encoded by nucleotides 518-526 (see Fig. 3 underlined). No homology was found to sequences in the Genpept 71.0, EMBL 30.0 and Swiss-Prot 21 databases. The predicted molecular weight of the translated polypeptide was 23,865 daltons and 22,177 daltons without the leader sequence.

Example 3 - Nature of the allergen Der p VII in mite extracts

As a first step to identifying the Der p VII native protein allergen, a pool of allergic serum obtained as described previously was absorbed with an equal volume of pGEX-1 HD6 lysate or a control vector lysate (Greene and Thomas Molec. Immunol. (1992) 29:259-262). The serum was then used for IgE Western blotting of house dust mite extracts separated by

SDS-PAGE performed according to Laemmli (Laemmli, U.K., Nature (1970) 227:680-5) with an 8-18% gradient in 10-12 cm gel assemblies or 13% mini protean II apparatus (Bio-Rad, Richmond, VI, USA). For dust mite extracts, samples were loaded at 0.1 mg protein/track. For bacteria, cultures were centrifuged and the pellets suspended at 0.01 of the culture volume and 10 μ l added to sample buffer for electrophoresis. Purified proteins were electrophoresed at 2-5 μ l/track. Compared to the serum absorbed with vector control (Fig. 4, lane 2), the HD6 fusion protein absorbed serum (Fig. 4, lane 1) showed a loss of reactivity to bands with Mr of 29, 27 and 11.5 K.

To examine this further, rabbit antibodies to the HD6 protein were affinity purified from a hyperimmune serum using nitrocellulose filters lifted from plates confluent with λ gt11-HD6 plaques. Briefly, antibodies with specificities for the allergen expressed by the λ gt11 clones were isolated from a hyperimmune rabbit anti-*D. pteronyssinus* serum (Greene, W.K., et al., Int Arch Allergy Appl Immunol (1990) 92:30-8) (produced by repeated injections into a rabbit of mite extract) by affinity purification using a nitrocellulose filter blotted on a plaque lawn (Ozaki, L.S., et al., J. Immunol Methods (1986) 89:213-9) as the absorbant. λ gt11 derived phage (clone HD6) were plated at 10 000 pfu per 90 cm petri dishes and overlaid with nitrocellulose saturated with isopropyl- β -D-thiogalactopyranoside (IPTG) under the same conditions used to screen the library. After overnight incubation, the filter was flipped to expose the other side to the lawn and incubated for 2 hours at 37°C. The filter was then washed in the TNT buffer, (0.01 M Tris hydrochloride, 0.15 NaCl, 0.05% Tween 20 v/v pH 8.0). One ml of rabbit antiserum which had been incubated overnight in 1 ml of a lysate of λ gt11 lysogen was diluted to 20 mls with TNT and skim milk powder added to 5%. Aliquots of 5 ml were then rocked in petri dishes containing the filters for 1 hour at room temperature. The filters were washed three times in TNT and incubated for 15 minutes at room temperature in 0.1 M glycine, 0.15 M NaCl pH2.6 to elute the antibodies. Each 5 ml eluate was then neutralized by adding 650 μ l of 100 mM Tris and 1.5 m NaCl, 50 ml 1% sodium azide and 0.25 g skim milk. The solution was dialysed against PBS.

The affinity purified antibody was then absorbed with *E. coli* lysate used to develop Western blots as described, on the house dust mite extract and found to react with bands of Mr 29, 27 and 24 (Fig. 5). The specificity of the reactivity was further checked by absorbing the affinity purified antibodies with a pGEX-HD6 lysate expressing the protein (Fig. 5, lane 3) or a control pGEX construct, pGEX-D15 (Fig. 5, lane 2). The serum absorbed with HD6 (lane 3) lost reactivity to all bands. The affinity purification therefore shows that antibodies to the allergen have specificities for components at Mr 29, 27 and 24 K. The same pattern of multiple binding to that described above with extracts prepared from CSL mites was also found with another extract from Hollister-Stier Laboratories, Spokane, WA, USA.

1002455-121901

The finding that antibodies to the HD6 lysate reacted specifically to at least 3 bands on Western blotting has implications for determining the number of allergens recognized by individual mite allergic patients. The multiple bands were found for the two independent extracts examined and the absorption studies with allergic serum showed that the 29 and 27 K bands had IgE reactivity and that this recombinant molecule appeared to absorb out all of the reactivity to each band. It is not, however, known from this investigation if all patients react with each band. Because the Western analysis was performed using reducing conditions and the bands had Mr greater than that calculated from the translated sequence, the different forms of the allergens may be interpreted as different glycosylation products. This can be confirmed with some caution taken to control for denaturation by the deglycosylation procedures. The pattern nevertheless indicates that the number of allergic specificities is less than that indicated by electrophoretic procedures, a significant observation for immunotherapeutic strategies using purified, recombinant or peptide allergens. Alternatively, the different Mr bands reacting with the anti-HD6 antibody may indicate the presence of related or cross reactive allergens.

Example 4 - Isolation of a cDNA clone encoding Der f VII from a λ gt11 cDNA library

A λ gt11 cDNA library was prepared from live adult *Dermatophagoides farinae* purchased from the Commonwealth Serum Laboratories, Parkville Australia (Thomas, W., et al., Int Arch Allergy Appl Immunol (1988) 85:127-9). The library was prepared according to Trudinger et al., (1991) Chem. Exp. Allergy, 21:33-37).

PCR amplification and DNA sequencing were used to isolate Der f VII cDNA from the λ gt11 library. An oligonucleotide primer (Df1 in Table 1) based on the predicted N-terminal sequence of Der p VII was made. This primer had the sequence GCGAATTCGATCCAATTCACTATGAT-3' (SEQ ID NO: 8). The first GCGAATTC encodes an EcoRI site (GAATTC) and the sequence GAT encodes the first six residues of Der p VII. For the other primer, the λ gt11 GGTGGCGACGACTCCTGGAGCCCCG-3' (SEQ ID NO: 9) forward primer (Df2 in Table 1) flanking the EcoRI cloning site was used (New England Biolabs, Beverly, U.S.A.).

The PCR reactions were carried out in a final reaction volume of 50 μ l containing 20 mM Tris-HCl pH 8.2, 10 mM KCl, 6 mM (NH₄)₂SO₄, 2mM MgCl₂, 0.1% Triton X-100, 10 ng μ nuclease-free BSA, 10 mM dNTPs, 20 pmol of each primer and 2.5 units of Pfu DNA polymerase. This was obtained as a kit from Stratagene (La Jolla, California, U.S.A.). Target DNA (λ gt11 *D. farinae* cDNA ligations, 0.001 μ g) was added and the contents of the tube were mixed and overlayed with paraffin oil. The tubes were initially denatured at 95°C for 5 min., then annealed at 55°C for 2 min. and extended at 72°C for 2

min. Thereafter for 48 cycles, denaturing was carried out for 1 min. at 94°C and annealing for 1 min. at 55°C and extension as before. In the final (50th) cycle, the extension reaction was increased to 10 min. to ensure that all amplified products were full length.

5 Ten microlitres of the reaction were then checked for amplified bands on a 1% agarose gel. The remainder of the reaction mixture was ethanol precipitated prior to purification of the amplified product on a low melting point agarose gel (Bio-Rad., Richmond, U.S.A.).

10 The purified PCR product was digested with EcoRI and was ligated into the M13 vector mp18 (see Messing, *supra*), digested with EcoRI and then transferred into *E. coli* strain TG1 competent cells. Isolated white plaques were picked and used to prepare phage stocks and single-stranded DNA for sequencing.

Example 5- DNA Sequence Analysis of Der f VII cDNA

15 DNA sequencing was performed with the dideoxynucleotide chain termination method using Sequenase version 2.0 (USB Corp., Cleveland, U.S.A.) according to the supplier's protocol. The primers used for sequencing include the M13 sequencing primer (-40) a 17-mer GTTTTCCCAGTCACGAC-3' (SEQ ID NO: 10) (Df3 in Table 1), the primer Df1 (SEQ ID NO: 8) used for PCR reaction described in Example 4, and 2 other
20 oligonucleotide primers, Df4 and Df5, both shown in Table 1. The primer Df4 GGTGAATTAGCCATGCG-3' (SEQ ID NO: 11) was previously used for the sequencing of Der p VII and primer Df5 TCAATCTTGGATCCAATTTTGGC-3' (SEQ ID NO: 12) was based on sequences of Der f VII from nucleotides 559-582.

To isolate a cDNA containing the 5' untranslated region of Der f VII, an
25 oligonucleotide primer based on the C terminal sequence of Der f VII was made. This primer (Df6 in Table 1) had the sequence GGAATTCTTAATTTTTTTTCCAATTCACG-3' (SEQ ID NO: 13). The first GGAATTC encodes a EcoRI site. This sequence and the following sequence (TTA . . .). are complementary to the reverse sequences of the stop codon and the last six residues of Der f VII. For the other primer, the λ gt11
30 TTGACACCAGACCAACTGGTAATG-3' reverse primer (SEQ ID NO: 14) (Df7 in Table 1), flanking the EcoRI cloning site, was used.

The PCR reactions were carried out according to conditions described in Example 4. The PCR product was purified on a low melting point agarose gel, digested with EcoRI and was ligated into pUC 19 digested with EcoRI and then transferred into *E. coli* strain
35 TGI competent cells. Plasmid DNA from transformant *E. coli* was isolated and used for sequencing.

10024955-121901

DNA sequencing was performed using the same dideoxynucleotide chain termination method, Sequence version 2.0, described above. However, before sequencing, the double-stranded plasmid DNA templates were denatured by treatment with NaOH and neutralized by the addition of sodium acetate and then ethanol precipitated according to the supplier's protocol. To obtain the 5' untranslated end of Der f VII cDNA, the primer Df8 (see Table 1) was used. This primer had the sequence ATGACGTTCTGAATTTATC-3' (SEQ ID NO: 15) which corresponds to the reverse sequence of Der f VII from nucleotide no. 225-208.

Table 1. Oligonucleotides used for PCR amplification and sequencing

<u>Name</u>	<u>Sequence</u>	<u>derived from Der f VII</u> <u>nucleotide positions</u>
Df1	GCGAATTCGATCCAATTCATGAT-3'	94-111
Df2	GGTGGCGACGACTCCTGGAGCCCG-3'	lambda gt11 forward primer
Df3	GTTTTCCCAGTCACGAC-3'	M13 sequencing primer (-40)
Df4	GGTGAATTAGACATGCG-3'	247-263
Df5	TCAATCTTGGATCCAATTTTGGC-3'	559-582
Df6	CGAATTCTTAATTTTTTCCAATTCACG-3'	684-664
Df7	TTGACACCAGACCAACTGGTAATG-3'	lambda gt11 reverse primer
Df8	ATGACGTTCTGAATTTATC-3'	225-208

Equivalents

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are considered to be within the scope of this invention and are encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

10

- (A) NAME: WESTERN AUSTRALIAN RESEARCH INSTITUTE FOR CHILD HEALTH
- (B) STREET: GPO Box D184
- (C) CITY: Perth
- (D) STATE: MA
- (E) COUNTRY: Western Australia
- (F) POSTAL CODE (ZIP): 6001

15

- (ii) TITLE OF INVENTION: Allergenic Protein and Peptides From
House Dust Mite and Uses Therefor

20

- (iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

25

- (A) ADDRESSEE: Lahive & Cockfield
- (B) STREET: 60 State Street
- (C) CITY: Boston
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) ZIP: 02109

30

(v) COMPUTER READABLE FORM:

35

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

40

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

45

- (A) APPLICATION NUMBER: USSN 08/031,141
- (B) FILING DATE: 12 March 1993
- (c) APPLICATION NUMBER: USSN 08/081,540
- (D) FILING DATE: 22 JUNE 1993

(viii) ATTORNEY/AGENT INFORMATION:

50

- (A) NAME: Amy E. Mandragouras
- (B) REGISTRATION NUMBER: 36,207
- (C) REFERENCE/DOCKET NUMBER: 053.2 PCT (IMI-032CPPC)

(ix) TELECOMMUNICATION INFORMATION:

55

- (A) TELEPHONE: 617-227-7400
- (B) TELEFAX: 617-227-5941

10004955-121001

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 812 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 68..712

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

20	TTTTTTTTTT TTTTGGTTAT TCCCATTTTT TTCATATCGT AAAAATCCAA ATTCACTTTT	60
	TTACCAA ATG ATG AAA TTA TTA TTG ATT GCT GCC GCA GCT TTT GTT GCC	109
	Met Met Lys Leu Leu Leu Ile Ala Ala Ala Ala Phe Val Ala	
25	-1 -5 -10	
	GTT TCG GCT GAT CCA ATT CAC TAT GAT AAA ATC ACC GAA GAA ATT AAC	157
	Val Ser Ala Asp Pro Ile His Tyr Asp Lys Ile Thr Glu Glu Ile Asn	
	-15 1 5 10	
30	AAA GCT GTT GAT GAA GCC GTC GCT GCA ATT GAA AAA TCC GAA ACA TTC	205
	Lys Ala Val Asp Glu Ala Val Ala Ala Ile Glu Lys Ser Glu Thr Phe	
	15 20 25	
35	GAT CCA ATG AAG GTA CCC GAT CAT TCT GAT AAA TTC GAA CGA CAT ATT	253
	Asp Pro Met Lys Val Pro Asp His Ser Asp Lys Phe Glu Arg His Ile	
	30 35 40 45	
40	GGT ATC ATC GAT TTA AAA GGT CAA TTA GAC ATG CGA AAC ATT CAA GTT	301
	Gly Ile Ile Asp Leu Lys Gly Gln Leu Asp Met Arg Asn Ile Gln Val	
	50 55 60	
	CGA GGA TTA AAA CAA ATG AAA CGT GTA GGT GAT GCT AAT GTG AAA AGT	349
	Arg Gly Leu Lys Gln Met Lys Arg Val Gly Asp Ala Asn Val Lys Ser	
45	65 70 75	
	GAA GAT GGT GTT GTC AAA GCT CAT TTG TTG GTC GGT GTT CAT GAT GAC	397
	Glu Asp Gly Val Val Lys Ala His Leu Leu Val Gly Val His Asp Asp	
	80 85 90	
50	GTT GTT TCA ATG GAA TAT GAT TTA GCA TAC AAA TTG GGT GAT CTT CAT	445
	Val Val Ser Met Glu Tyr Asp Leu Ala Tyr Lys Leu Gly Asp Leu His	
	95 100 105	
55	CCA AAC ACT CAT GTC ATT TCG GAT ATT CAG GAT TTT GTT GTC GAA TTA	493

10024955-121901

Pro Asn Thr His Val Ile Ser Asp Ile Gln Asp Phe Val Val Glu Leu
110 115 120 125

5 TCG CTC GAA GTT AGC GAA GAA GGT AAT ATG ACA TTG ACA TCG TTC GAA 541
Ser Leu Glu Val Ser Glu Glu Gly Asn Met Thr Leu Thr Ser Phe Glu
130 135 140

10 GTA CGT CAA TTT GCC AAT GTT GTC AAT CAT ATT GGT GGT CTT TCA ATT 589
Val Arg Gln Phe Ala Asn Val Val Asn His Ile Gly Gly Leu Ser Ile
145 150 155

15 TTG GAT CCA ATT TTC GCT GTC TTA TCC GAT GTT TTG ACC GCT ATT TTC 637
Leu Asp Pro Ile Phe Ala Val Leu Ser Asp Val Leu Thr Ala Ile Phe
160 165 170

CAG GAT ACC GTA CGT GCA GAA ATG ACC AAA GTA TTG GCA CCA GCA TTC 685
Gln Asp Thr Val Arg Ala Glu Met Thr Lys Val Leu Ala Pro Ala Phe
175 180 185

20 AAA AAA GAA TTG GAA CGA AAC AAC CAA 715
Lys Lys Glu Leu Glu Arg Asn Asn Gln
190 195

25 TAGACTTACA CACAACATAA CACTGTTATT TTTACACTGG ATAATCAAAT GAAATAAATT 775
TTTTTATCAT TTTGTTTAAA AAAAAAAAAA AAAAAAAAAA 812

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 215 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

40 Met Met Lys Leu Leu Leu Ile Ala Ala Ala Ala Phe Val Ala
-1 -5 -10

Val Ser Ala Asp Pro Ile His Tyr Asp Lys Ile Thr Glu Glu Ile Asn
-15 1 5 10

45 Lys Ala Val Asp Glu Ala Val Ala Ala Ile Glu Lys Ser Glu Thr Phe
15 20 25

50 Asp Pro Met Lys Val Pro Asp His Ser Asp Lys Phe Glu Arg His Ile
30 35 40 45

Gly Ile Ile Asp Leu Lys Gly Gln Leu Asp Met Arg Asn Ile Gln Val
50 55 60

10024955-121901

Arg Gly Leu Lys Gln Met Lys Arg Val Gly Asp Ala Asn Val Lys Ser
65 70 75

5 Glu Asp Gly Val Val Lys Ala His Leu Leu Val Gly Val His Asp Asp
80 85 90

Val Val Ser Met Glu Tyr Asp Leu Ala Tyr Lys Leu Gly Asp Leu His
95 100 105

10 Pro Asn Thr His Val Ile Ser Asp Ile Gln Asp Phe Val Val Glu Leu
110 115 120 125

Ser Leu Glu Val Ser Glu Glu Gly Asn Met Thr Leu Thr Ser Phe Glu
15 130 135 140

Val Arg Gln Phe Ala Asn Val Val Asn His Ile Gly Gly Leu Ser Ile
145 150 155

20 Leu Asp Pro Ile Phe Ala Val Leu Ser Asp Val Leu Thr Ala Ile Phe
160 165 170

Gln Asp Thr Val Arg Ala Glu Met Thr Lys Val Leu Ala Pro Ala Phe
25 175 180 185

Lys Lys Glu Leu Glu Arg Asn Asn Gln
30 190 195

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATCCAATTC ACTATGAT

18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5 GGTGAATTAG ACATGCG

17

(2) INFORMATION FOR SEQ ID NO:5:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCAATTTTGG ATCCAATTTT CGCT

24

25 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 761 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 43..681

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCTTATAT CAATAACAAT CCAAAAAAAC ATATCTTACA AA ATG ATG AAA TTT
Met Met Lys Phe

54

45

TTG TTG ATT GCT GCC GTG GCA TTT GTC GCC GTT TCG GCT GAT CCA ATT
Leu Leu Ile Ala Ala Val Ala Phe Val Ala Val Ser Ala Asp Pro Ile
5 10 15 20

102

10024955-121901

100455-121901

	CAC TAT GAT AAA ATC ACC GAA GAA ATC AAC AAA GCT ATT GAT GAT GCC	150
	His Tyr Asp Lys Ile Thr Glu Glu Ile Asn Lys Ala Ile Asp Asp Ala	
	25 30 35	
5	ATT GCT GCT ATT GAA CAA TCC GAA ACA ATA GAT CCA ATG AAA GTA CCT	198
	Ile Ala Ala Ile Glu Gln Ser Glu Thr Ile Asp Pro Met Lys Val Pro	
	40 45 50	
10	GAT CAT GCC GAT AAA TTC GAA CGT CAT GTT GGT ATT GTG GAT TTC AAA	246
	Asp His Ala Asp Lys Phe Glu Arg His Val Gly Ile Val Asp Phe Lys	
	55 60 65	
15	GGT GAA TTA GCC ATG CGA AAC ATT GAG GCT CGA GGA TTG AAA CAA ATG	294
	Gly Glu Leu Ala Met Arg Asn Ile Glu Ala Arg Gly Leu Lys Gln Met	
	70 75 80	
20	AAA CGT CAA GGT GAT GCT AAT GTC AAA GGT GAA GAG GGT ATT GTT AAA	342
	Lys Arg Gln Gly Asp Ala Asn Val Lys Gly Glu Glu Gly Ile Val Lys	
	85 90 95 100	
25	GCT CAT TTG TTG ATC GGT GTT CAC GAT GAT ATC GTC TCG ATG GAA TAT	390
	Ala His Leu Leu Ile Gly Val His Asp Asp Ile Val Ser Met Glu Tyr	
	105 110 115	
30	GAT TTA GCA TAC AAA TTG GGT GAT CTT CAT CCA ACC ACT CAT GTC ATT	438
	Asp Leu Ala Tyr Lys Leu Gly Asp Leu His Pro Thr Thr His Val Ile	
	120 125 130	
35	TCG GAT ATT CAA GAT TTT GTT GTT GCC TTG TCC CTT GAA ATT TCT GAT	486
	Ser Asp Ile Gln Asp Phe Val Val Ala Leu Ser Leu Glu Ile Ser Asp	
	135 140 145	
40	GAA GGT AAC ATA ACA ATG ACA TCT TTT GAA GTA CGA CAA TTC GCT AAT	534
	Glu Gly Asn Ile Thr Met Thr Ser Phe Glu Val Arg Gln Phe Ala Asn	
	150 155 160	
45	GTT GTC AAC CAT ATT GGT GGT CTT TCA ATC TTG GAT CCA ATT TTT GGC	582
	Val Val Asn His Ile Gly Gly Leu Ser Ile Leu Asp Pro Ile Phe Gly	
	165 170 175 180	
50	GTT TTA TCT GAT GTA TTG ACC GCT ATT TTC CAA GAC ACC GTA CGT AAG	630
	Val Leu Ser Asp Val Leu Thr Ala Ile Phe Gln Asp Thr Val Arg Lys	
	185 190 195	
55	GAA ATG ACC AAA GTA TTG GCA CCA GCA TTT AAA CGT GAA TTG GAA AAA	678
	Glu Met Thr Lys Val Leu Ala Pro Ala Phe Lys Arg Glu Leu Glu Lys	
	200 205 210	
50	AAT TAACCAATAG ACATCATTTT TCCAACGTGA CAATCTCTAT TTCCTGACA	731
	Asn	
	ATAAAATAAA ATTTTATTT TTATTTCTCC	761

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 213 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Met Lys Phe Leu Leu Ile Ala Ala Val Ala Phe Val Ala Val Ser
1 5 10 15

Ala Asp Pro Ile His Tyr Asp Lys Ile Thr Glu Glu Ile Asn Lys Ala
20 25 30

Ile Asp Asp Ala Ile Ala Ala Ile Glu Gln Ser Glu Thr Ile Asp Pro
35 40 45

Met Lys Val Pro Asp His Ala Asp Lys Phe Glu Arg His Val Gly Ile
50 55 60

Val Asp Phe Lys Gly Glu Leu Ala Met Arg Asn Ile Glu Ala Arg Gly
65 70 75 80

Leu Lys Gln Met Lys Arg Gln Gly Asp Ala Asn Val Lys Gly Glu Glu
85 90 95

Gly Ile Val Lys Ala His Leu Leu Ile Gly Val His Asp Asp Ile Val
100 105 110

Ser Met Glu Tyr Asp Leu Ala Tyr Lys Leu Gly Asp Leu His Pro Thr
115 120 125

Thr His Val Ile Ser Asp Ile Gln Asp Phe Val Val Ala Leu Ser Leu
130 135 140

Glu Ile Ser Asp Glu Gly Asn Ile Thr Met Thr Ser Phe Glu Val Arg
145 150 155 160

Gln Phe Ala Asn Val Val Asn His Ile Gly Gly Leu Ser Ile Leu Asp
165 170 175

Pro Ile Phe Gly Val Leu Ser Asp Val Leu Thr Ala Ile Phe Gln Asp
180 185 190

Thr Val Arg Lys Glu Met Thr Lys Val Leu Ala Pro Ala Phe Lys Arg
195 200 205

10024955.121901

Glu Leu Glu Lys Asn
210

5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

20 GCGAATTCGATCCAATTCACATATGAT 26

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

35 GGTGGCGACGACTCCTGGAGCCCCG 24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

50 GTTTTCCCAGTCACGAC 17

10024955.121901

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGTGAATTAGACATGCG

17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCAATCTTGGATCCAATTTTGGC

24

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAATTCTTAATTTTTTTTCCAATTCACG

28

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10024955.121901
T05T2T-554200T

(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTGACACCAGACCAACTGGTAATG

24

10 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATGACGTTCTGAATTTATC

18

1004955-12194